Evolution of entomopathogenicity in fungi

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ARTICLE INFO

ABSTRACT

The recent completions of publications presenting the results of a comprehensive study on the fungal phylogeny and a new classification reflecting that phylogeny form a new basis to examine questions about the origins and evolutionary implications of such major habits among fungi as the use of living arthropods or other invertebrates as the main source of nutrients. Because entomopathogenicity appears to have arisen or, indeed, have lost multiple times in many independent lines of fungal evolution, some of the factors that might either define or enable entomopathogenicity are examined. The constant proximity of populations of potential new hosts seem to have been a factor encouraging the acquisition or loss of entomopathogenicity by a very diverse range of fungi, particularly when involving gregarious and immobile host populations of scales, aphids, and cicadas (all in Hemiptera). An underlying theme within the vast complex of pathogenic and parasitic ascomycetes in the Clavicipitaceae (Hypocreales) affecting plants and insects seems to be for interkingdom host-jumping by these fungi from plants to arthropods and then back to the plant or on to fungal hosts. Some genera of Entomophthorales suggest that the associations between fungal pathogens and their insect hosts appear to be shifting away from pathogenicity and towards nonlethal parasitism.

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1. Introduction and first principles

Whether the earliest fungi were pathogens or saprobes has been an intriguing mycological question (Savile, 1968). The resolution of which nutritional habit (i.e., which preferred source of nutrients) came first among the fungi remained speculative until the completion of a recent phylogenetic analysis of the fungi using synthesized sequence data from six genes from an extensive sampling from all major groups of fungi (James et al., 2006). In view of these new studies, whether the earliest fungi were saprobes or parasites is a question that is neither answerable nor important for understanding the biologies of extant fungal taxa regardless of the sources from which they obtain their nutrient requirements.

Despite the number and diversity of fungi included in the James et al. (2006) phylogenetic survey, it still does not seem to be sufficiently large or comprehensive to draw any conclusions about how the earliest fungi obtained their nutrition. Fungal parasitism has arisen independently and repeatedly in many different lines of fungal evolution. The discussion presented here uses the new fungal classifications (Hibbett et al., 2007; Sung et al., 2007) that reflect the phylogeny reported by James et al. (2006) because it embodies the most trustworthy hypothesis about the relatedness of fungi in each of the phyla, classes and

orders now being recognized (Table 1 lists the taxa in this new classification relevant to this discussion).
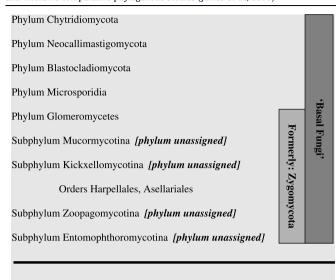
1.1. The new phylogenetically based classification of Fungi

Some higher levels of the new fungal classification do, indeed, represent groups in which all taxa show specific nutritional preferences such as being plant-pathogenic or lichenized fungi. These habits are especially common among the 'higher' fungi (Subkingdom Dikarya; the ascomycetes and basidiomycetes). Even though most higher fungi are associated with plants in some manner, the specific nutritional relationships between these fungi and plants range from virulent pathogens to decomposers of plant detritus to beneficial (e.g., in mycorrhizae or some endophytic associations).

The taxonomy of the more basal fungi–watermolds and zygomycetes—has historically been more complex, more problematic, and less intensively studied than for higher fungi. In Hibbett's et al. (2007) new classification, 'basal' fungi are dispersed among a much greater number and diversity of phyla than are the Dikarya. Among the basal fungal phyla, only the arbuscular mycorrhizal fungi of the Glomeromycota and the so-called 'rumen chytrids' of the Neocallimastigomycota show extreme nutritional specializations restricting them to very narrow ecological niches. Glomeromycetes are obligatorily associated with the roots of plant hosts. The anaerobic and amitochondrial zoosporic fungi in Neocallimastigomycota occur in the rumen or cecum of

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Table 1
A highly selected partial listing of phyla, classes and subclasses of the Kingdom Fungi using the classification of Hibbett et al. (2007) that was, in turn, based on extensive and intensive comparative phylogenetic studies (James et al., 2006)

Phylum Chytridiomycota	
Phylum Neocallimastigomycota	
Phylum Blastocladiomycota	
Phylum Microsporidia	'Basal Fungi'
Phylum Glomeromycetes	
Subphylum Mucormycotina *[phylum unassigned]*	
Subphylum Kickxellomycotina *[phylum unassigned]*	Formerly: *Zygomycota*
Orders Harpellales, Asellariales	
Subphylum Zoopagomycotina *[phylum unassigned]*	
Subphylum Entomophthoromycotina *[phylum unassigned]*	

Subkingdom Dikarya

  Phylum Ascomycota

    Subphylum Pezizomycotina

      Class Eurotiomycetes

      Class Dothideomycetes

      Class Laboulbeniomycetes

      Class Lecanoromycetes *[lichenized fungi]*

      Class Orbiliomycetes

      Class Sordariomycetes

        Order Hypocreales

  Phylum Basidiomycota

    Subphylum Pucciniomycotina

      Class Pucciniomycetes *[rust fungi]*

        Order Septobasidiales

    Subphylum Ustilaginomycotina *[smut fungi]*

    Subphylum Agaricomycotina *['mushrooms, toadstools']*

herbivorous mammalian digestive systems (Li et al., 1993; Wubah et al., 1991a, b) or possibly also in some other anaerobic environments. Comparatively few entomopathogens now remain in the restricted Chytridiomycota, from which Neocallimastigomycota and Blastocladiomycota were segregated. Most entomopathogenic chytrids—mainly the species of *Coelomomyces* (Couch and Bland, 1985) and *Coelomycidium* (Debaisieux, 1920)—are now in the Blastocladiomycota, a group with high tolerance for survival in dry environments and life histories that usually include alternation of haploid and diploid generations.

Microsporidia are the most recent major additions to the fungal world (see Keeling and Fast, 2002) but these obligatorily intracellular parasites that affect a wide range of invertebrates and verte-

brates present too many unsolved biological puzzles for them to be considered further here.

The zygomycetes as known previously have been dispersed into the Glomeromycota and four other phylogenetic lineages treated as new subphyla–Mucormycotina, Kickxellomycotina, Zoopagomycotina, and Entomophthoromycotina—with unresolved phyletic affinities. These newly disaffiliated subphyla may still be recognized as four separate phyla or fewer if new evidence indicates any need for regrouping but any and all of these phyla will have to be described as new. *Sporodiniella umbellata* is the only taxon in the Mucormycotina consistently associated with insects as a pathogen (Evans and Samson, 1977). Within the Kickxellomycotina, the orders Kickxellales and Dimargaritales are mostly mycoparasitic on mucoroid zygomycetes, and the Harpellales and Asellariales comprise endocommensals in the hindguts of arthropods. The class Trichomycetes (Lichtwardt et al., 2001) was formally abandoned long ago when the Harpellales and Asellariales were recognized to be zygomycetes allied with the Kickxellales/Dimargaritales, and the Eccrinales and Amoebidiales were understood to be protozoans rather than true fungi. The Dikarya (ascomycete and basidiomycete fungi) are most closely related to the Glomeromycota among the basal fungi, and this may account for the very much stronger nutritional associations of these fungi with plants rather than animals.

The new phylogenetic perspective of fungal relationships (Fig. 1 in James et al., 2006) makes it clear that such fungal nutritional habits as parasites or pathogens affecting plants or animals, as mycoparasites, as saprobes, or as mycorrhizae show no clear sign of having arisen once and then to be passed down unaltered to all later descendants in that lineage. With reference to the taxa listed in Table 1, the entomopathogenic or entomoparasitic habit occurs most prominently in the Blastocladiomycota (*Coelomomyces* spp, *Coelomycidium simulii*), the Entomophthoromycotina (with entomopathogens in five of its six families and with the great majority of genera being obligately entomopathogenic; Humber, 1989; Bałazy, 1993; Keller and Petrini, 2005) the Kickxellomycotina (endocommensal Harpellales and Asellariales), Eurotiomycetes (*Ascosphaera* and other genera), Laboulbeniomycetes (ectoparasitic ascomycetes with determinate thallic growth), Dothideomycetes (*Myriangium*), Sordariomycetes (mostly in Hypocreales), and Pucciniomycetes (*Septobasidium* and its relatives). Still more fungi in the Zoopagomycotina, Entomophthoromycotina, Orbiliomycetes, and Sordariomycetes have obligatory parasitic relationships with microinvertebrates outside the Arthropoda such as nematodes, tardigrades, amoebae, rotifers, and more. Even prior to a detailed reconstruction of fungal phylogeny (James et al., 2006), it was obvious from the wide and seemingly random taxonomic distribution of fungi pathogenic or parasitic for insects or other invertebrate hosts that these nutritional habits emerged repeatedly and independently in many different fungal lineages.

2. Origins of entomopathogenicity

2.1. Pathogenicity for insects

Even within the Hypocreales, the order with the largest number and diversity of fungal taxa attacking invertebrates, there is reason to believe that the entomopathogenic habit has multiple origins. The traditional concept of the family Clavicipitaceae (Hypocreales; e.g., Kobayasi, 1941; Rogerson, 1970; White et al., 2003) has always been problematic because it included both a large number and diversity of genera whose primary hosts are plants (Bischoff and White, 2003) as well as the comparatively few teleomorphic genera (although the broadly described *Cordyceps* included

hundreds of species; see Kobayasi, 1941; Sung et al., 2007) and a bewildering array of anamorphic (conidial) taxa affecting insects (Hodge, 2003) or nematodes and other microinvertebrates (Gams and Zare, 2003). In the latest taxonomy of the Clavicipitaceae, Sung et al. (2007) segregate the family by splitting off the Cordycipitaceae and Ophiocordycipitaceae; major interkingdom host shifts have occurred in each of these three families. The Clavicipitaceae is typified by the phytopathogenic genus *Claviceps*, and most genera and species in this newly restricted family are phytophagous. Nonetheless, this family in its newly restricted sense also includes many entomopathogenic teleomorphs classified in *Hypocrella*, *Metacordyceps* (Sung et al., 2007), *Regiocrella* (Chaverri et al., 2005), and *Torrubiella*; entomopathogenic anamorphs from this family are classified in *Aschersonia*, *Metarhizium*, *Nomuraea*, *Paecilomyces*-like fungi excluded from *Isaria* sensu stricto (Luangsa-ard et al., 2004, 2005), some *Verticillium*-like fungi not reassigned since the reclassification of *Verticillium* sect. *Prostrata* (Zare et al., 2000, 2001; Gams and Zare, 2001; Sung et al., 2001; Zare and Gams, 2001a, b), and the species of *Pochonia* and *Rotiferophthora* affecting nematodes and rotifers, respectively (Zare et al., 2001). The entomopathogens of *Hypocrella*/*Aschersonia* group and the seed-parasitic genus *Shimizuomyces* share a branch with the plant pathogens of the Clavicipitaceae whereas species from microinvertebrates (with anamorphs in *Pochonia*, *Tolypocladium*, and *Rotiferophthora*) and entomopathogens (with anamorphs in *Metarhizium* and *Nomuraea*) occupy an altogether separate branch within this family (Sung et al., 2007). The newly validated (and also restricted) family Cordycipitaceae appears to be wholly entomopathogenic and includes teleomorphs in the highly speciose genera *Cordyceps* and *Torrubiella* and other genera with comparatively few species as well as anamorphs in genera such as *Beauveria* and the morphologically similar *Microhilum*, *Engyodontium*, *Isaria*, *Mariannaea*-like species, *Lecanicillium*, and *Simplicillium*. The greatest diversity of nutritional habits may occur in the new family Ophiocordycipitaceae with two teleomorphic genera (segregated from *Cordyceps*)—*Ophiocordyceps* (entomopathogens) and *Elaphocordyceps* (a mix of entomopathogens, nematode pathogens, and mycopathogens)—and anamorphs dispersed among *Haptocillium*, *Harposporium*, *Hirsutella*, *Hymenostilbe*, some *Paecilomyces*-like species (excluded from *Isaria* and the Cordycipitaceae), *Paraisaria*, *Sorosporella*, *Syngliocladium*, *Tolypocladium*, and some *Verticillium*-like species (not reclassified since the revision of *Verticillium* sect. *Prostrata* noted above).

Entomophthoralean fungi have not yet received such detailed genomic study as the clavicipitoid fungi. However, all evidence based on traditional characters (Humber, 1984), sterol spectra (Weete and Gandhi, 1997), and gene sequence data (James et al., 2006; Huang, Hodge and Humber, unpublished) confirm that the Entomophthorales occupies a basal position among zygomycetes, and that within this order the most basal taxa are the primarily saprobic genera in the families Basidiobolaceae and Anyclistaceae (especially *Conidiobolus*, but some species in this genus are entomopathogenic). The Entomophthoraceae and Neozygitaceae seem to represent separate lineages within this order and display widely divergent biologies, but all taxa in both families are arthropod pathogens, and probably evolved this habit independently. Nutritional habits within the basal families of the order, Basidiobolaceae and Ancylistaceae, vary from mostly saprobes to saprobes capable of causing facultative vertebrate mycoses to facultative and obligatory entomopathogens, and even to a group of obligatory phytopathogens (*Ancylistes* species, all of which are pathogens of desmid algae). The Meristacraceae appears to be derived from the Ancylistaceae, but its taxa are known primarily from nematodes and tardigrades. The monotypic family Completoriaceae (*Completoria complens*) is known only as an intracellular parasite of fern gametophytes. The analysis of the general biology of these fungi (Humber, 1984) suggesting that entomopathogenicity may be the most derived nutritional habit in each lineage of the order has been largely corroborated by the PCR-based phylogenetic studies that have been done on these fungi (Jensen et al., 1998; Nielsen et al., 2001; James et al., 2006; Huang, Hodge and Humber, unpublished).

2.2. What sets entomopathogens apart?

Since there is no underlying phylogenetic linkage that suggests that entomopathogenicity arose once and has been retained, modified or subsequently lost by all later taxa in a given lineage, it becomes necessary to find other common properties shared by entomogenous fungi that might explain the enablement of diverse fungi to take advantage of the grand banquet of insects, other arthropods or, in its most generalized state, of other microinvertebrates as potential sources of nutrients.

The second major consideration is that lists of fungal pathogens of insects or other major invertebrates (e.g., the listings of the ARSEF culture collection, <http://arsef.fpsnl.cornell.edu>, or the Ecological Database of the World's Insect Pathogens, <http://cricket.inhs.uiuc.edu/edwipweb/edwipabout.htm>) versus lists of fungal pathogens of plants (for which comparable compendia would be impracticably large; for example, see so 'limited' a listing as that by Farr et al., 1989) are notable for their being mutually exclusive with a few possible exceptions, for instance, of some *Fusarium* isolates from insects that may best be identified as species vastly better known from plants.

Examples of entomopathogenic fungi can be found in most major fungal groups from watermolds through the basidiomycetes, but many major groups of fungi have no known activity against arthropods or other invertebrates (Roberts and Humber, 1981). Fungi with darkly melanized hyphae and/or conidia are rarely harmful to insects but are phytopathogens or important decomposers of plant detritus. Anamorphic fungi producing their conidia inside distinct fruiting bodies (Coelomycetes with conidia formed in pycnidia) are almost exclusively phytopathogens. The ascomycete subclass Pleosporomycetidae (formerly Loculoascomycetes, whose species produce bitunicate asci in pseudothecia) includes few entomopathogens. The Eurotiomycetes produce globose asci in closed fruiting bodies (cleistothecia) and comprise innumerable saprobes and plant fungi but very few entomopathogens except for *Ascosphaera* spp, all of which cause chalkbrood disease in bees, and the comparatively rare facultative diseases caused by *Aspergillus* species (but notably *not* by species of the closely related *Penicillium*) in some lepidopterans (e.g., silkworm, *Bombyx mori*) in such highly dense and stressed populations as those in commercial rearings.

An ability to digest chitin-rich substrates, thereby possibly enabling the penetration of a host's cuticle, seems to unite virtually all entomopathogenic fungi. The ability to degrade high densities of chitin, however, may have allowed an underground host-jump of a group of *Cordyceps* species from cicadas to the subterranean ascomycetes (false or deer truffles; *Elaphomyces* spp) (Nikoh and Fukatsu, 2000; Fukatsu and Nikoh, 2003).

While the importance of chitinase secretion during fungal germination and initial penetration of a host has been widely assumed, experimental evidence suggests that protease digestion of the matrix in which cuticular chitin is embedded may be more important for successful host penetrations (St. Leger et al., 1987, 1996; Screen et al., 2001). In addition, other physiological factors contributing to entomopathogenicity must also be considered. The straminipilan oomycete *Lagenidium giganteum*, a commercialized mosquito pathogen, shows some higher ability to utilize the trehalose in the mosquito's blood than does the host (McInnis and Domnas, 1973). Trehalose is the key blood sugar for arthro-

pods and appears to be utilized by most entomopathogenic fungi, but trehalose is not routinely produced in plants. Fungal depletion of trehalose resources means that a mycotized insect is being starved during the final stages of fungal development.

2.3. Examples allowing strong inferences about evolutionary changes

A few examples offer some strong evidence about how certain nutritional habits may have been adopted by fungi associated with insects. In the first place, scale insects (especially hemipterans in the families Coccidae and Aleyrodidae) appear to be the insect hosts most affected by the greatest number and diversity of fungal pathogens (Humber, unpublished). And the scales seem to be followed closely by the number and prominence of very different sets of fungi affecting hemipterans in the Aphididae and Cicadidae. These hemipterans tend to occur in dense, substantially immobile populations feeding on aerial plant parts or underground on plant roots; such behaviors allow sustained periods of proximity between insects, fungi, and other potential sources or hosts that can provide opportunities for pathogenic fungi to make the adaptive jump from plant to insect or from insect to truffle hosts. These fungus/hemipteran associations offer some of the clearest insights into the evolution of entomopathogenicity.

The growth of the vast majority of entomopathogenic fungi is clearly limited by the supply of nutrients provided by the affected host individual. *Hirsutella thompsonii*, for example, forms sparse conidia on a few wispy hyphae spreading across a plant surface from the smallest hosts (phyllocoptrutid and eriophyid rust mites) affected by this pathogen, and mycotized mites are difficult to detect. In culture, however, on the comparatively unlimited nutrients of any of a wide range of media, *H. thompsonii* grows and sporulates luxuriantly (Sampson et al., 1980). Most *Hypocrella* species (Hypocreales) form small stromata using only the nutrients available from the one to few scale insects under each stroma; however, a few *Hypocrella* species produce gigantic stromata (Hywel-Jones and Samuels, 1998) that cannot form without sustained nutrient inputs from the host plants long past the point where the mycotized host scales were obliterated (and a sequential shift for the fungal colony from entomopathogen to plant parasite once the underlying insect host is destroyed). Other genera of Hypocreales that begin their development as pathogens of scale insects but then go beyond the limitations of the scales to produce gigantic stromata include *Ascopolyporus* (Bischoff et al., 2005) and *Dussiella* (Koroch et al., 2004). *Hyperdermium* Sullivan et al. (2000) is closely related to both *Ascopolyporus* and even to *Cordyceps militaris* but appears to derive its nutrients exclusively from plants without any scale insects interposed between the host plant and fungus. What is not apparent about these genera is whether (1) the fungus is a scale pathogen that evolves increasingly larger stromata and becomes (secondarily?) dependent on obtaining further nutrients from the plants through the stylets of the affected scales as demonstrated for *Dussiella* (Koroch et al., 2004), or (2) whether these taxa with such large stromata predate those with the smaller stromata that would result from increasing restriction to the use of the nutrient resources of only the affected scale insects while decreasing or eliminating any need for continuous nutrient transfer from a host plant through an interposed scale insect.

The revised classification of the Clavicipitaceae (Sung et al., 2007) cannot distinguish between these possibilities but indicates that the scale fungi with large stromata are not a monophyletic group and, therefore, represent evolutionary convergences. *Hypocrella* and the similar scale-pathogenic genus *Regiocrella* (Chaverri et al., 2005) occupy a derived branch within the Clavicipitaceae while *Ascopolyporus* and *Hyperdermium* are in the Cordycipitaceae (most of whose members are entomogenous), and the familial placement of *Dussiella* is unresolved (Sung et al., 2007).

Phytocordyceps ninchukispora (Su and Wang, 1986) is a *Cordyceps*-like fungus with bizarre ascospores and grows exclusively on plants; nonetheless, its gene sequences led Sung et al. (2007) to conclude that this species is a highly derived species of *Cordyceps* that made the host-jump from insects to plants. The *Cordyceps* species mycoparasitic on subterranean, mycorrhizal ascocarps of *Elaphomyces* species are apparently derived by host-jumping from cicada-pathogenic *Cordyceps* species feeding on roots also colonized by *Elaphomyces* (Nikoh and Fukatsu, 2000; Fukatsu and Nikoh, 2003); these mycoparasites have been transferred to the new genus *Elaphocordyceps* (which also includes entomopathogens) in the new family Ophiocordycipitaceae (Sung et al., 2007).

The only significant basidiomycete-insect associations, those of *Septobasidium* species (Couch, 1938) with coccid scale insects, go far beyond the dependence on a scale's mouthparts to gain access to nutrients from the host plant's phloem as discussed above for hypocrealean scale pathogens with giant stromata. *Septobasidium* covers, protects, and allows increases of scale populations of scales while parasitizing a small proportion of apparently living scales with coiled haustoria that absorb nutrients from the host plant's phloem indirectly through the scale's hemocoel. Traditional and molecular characters place *Septobasidium* in a comparatively basal position among the rust fungi (Arun Kumar et al., 2007), all of whose members are otherwise obligatory phytopathogens; it is difficult to conceive that this unique fungus-scale-plant relationship could have arisen by any means except by the host transference of a phytopathogenic rust fungus onto parasitic scale insects on a shared plant host.

In the Entomophthoraceae (Entomophthorales), the species of *Massospora* (from periodical cicadas; Soper, 1974) and *Strongwellsea* (from muscoid fly adults; Humber 1976, 1982) may be evolving from the pathogenicity typical of all other genera in this family towards nonlethal parasitism since their development is restricted to part of the abdomen with an overall volume of fungal biomass that is small in comparison to the volume of the host body (Humber, 1982, 1984). Species in both of these genera sporulate from living hosts, even if by completely different mechanisms, while their cicada and fly hosts die sooner than healthy individuals of the affected populations. If a continued restriction of the overall growth in the host allowed these fungi to survive and to sporulate without shortening the host's normal lifespan, they would become parasites instead of pathogens.

The evidence about the evolution of entomopathogenicity among fungi indicates that this nutritional habit has arisen multiple times in disparate fungal groups, and that, once acquired, can be lost by cross-kingdom host-jumps to invertebrates outside the Arthropoda, to plants, or to other fungi. The full range and details of the evolutionary pressures that might affect the adoption of or switches among the saprobic, parasitic, pathogenic or any other type of specific nutritional habits remain unknown but invite hypotheses based on the biologies of extant fungi. Natural selective forces continue to operate in seemingly every conceivable direction as fungi adapt to the ever-changing physical and biological world in which they operate. The continual biological experiment of natural selection allows possible mechanisms for fungi to change their nutritional sources and even to start using nutrients from different kingdoms than at present. Natural selection may also lead a fungus to an increasing or decreasing level of nutritional and biological adjustment to its food source; such adjustments could move a fungus in any direction along the nutritional continuum from beneficial to commensal to saprobic to parasitic to pathogenic associations with the source of its nutrients. Stated metaphorically, the great "Perpetual Fungal Banquet and Heterotrophic Ball" continues: the rich assortment of dishes available may change or substitutions made with little notice; in the ballroom the styles of music being played will keep changing, and dancers may change partners at any time.

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